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Titel: Anvendelse af den regulatoriske sekvens for WAP genet til ekspression af heterologe gener i humane brystceller

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Use of the WAP or MMTV reglulat ry sequences for targeted expression of linked heterologous genes in human mammary cells, including human mammary carcinoma cells

The present invention relates to the use of the rodent WAP (Whey Acidic Protein) and the MMTV (Mouse Mammary Tumor Virus) regulatory sequences for targeted expression of linked DNA-sequences in human mammary carcinoma cells.

Background of the Invention

Mammary carcinoma is the most frequent tumour in women (Miller and Bulbrook, 1986). Up to now the conventional therapy involves surgical removal of the primary tumour followed by a chemo- or radiation therapy. Depending on the tumour stage, the rate of relapse is quite high and has a fatal outcome in most cases. A major problem is the elimination of all metastases and micrometastases. Both this, as well as the serious side effects for the patient caused by conventional treatment, favour the development of a gene therapy approach (for a review on gene therapy see Anderson, 1992). One such approach could involve the use of a modified retrovirus or retroviral vector, to specifically deliver therapeutic genes to mammary carcinoma cells. The therapeutic gene would either inhibit the proliferation of tumour cells or kill the tumour cells after infection (suicide or toxin genes). The great advantage of a viral system would be that the virus particles can be spread in the blood stream similarly to metastazising tumour cells, which will make it possible to eliminate micrometastases long before they can be detected by conventional methods. Systemic delivery however poses the problem of the ability to target the therapeutic retroviral vector's expression only to the tumour cells.Therefore a control element is required to ensure that the transferred retroviral vector is only active in tumour cells.

Retroviral vector systems consist of two components:

1) the retroviral vector itself is a modified retrovirus (vector plasmid) in which the genes encoding for the viral proteins have been replaced by therapeutic genes and/or marker genes to be transferred to the target cell. Since the replacement of th genes encoding for the viral proteins effectively cripples the virus it must be rescued

and/or marker genes to be transferred to the target cell. Since the replacement of the genes encoding for the viral proteins effectively cripples the virus it must be rescued by the second component in the system which provides the missing viral proteins to the modified retrovirus.

The second component is:

2) a cell line that produces large quantities of the viral proteins, however lacks the ability to produce replication competent virus. This cell line is known as the packaging cell line and consists of a cell line transfected with one or more plasmids carrying the genes enabling the modified retroviral vector to be packaged.

To generate the packaged vector, the vector plasmid is transfected into the packaging cell line. Under these conditions the modified retroviral genome including the inserted therapeutic and marker genes is transcribed from the vector plasmid and packaged into the modified retroviral particles (recombinant viral particles). This recombinant virus is then used to infect target cells in which the vector genome and any carried marker or therapeutic genes becomes integrated into the target cell's DNA. A cell infected with such a recombinant viral particle cannot produce new vector virus since no viral proteins are present in these cells. However the DNA of the vector carrying the therapeutic and marker genes is integrated in the cell's DNA and can now be expressed in the infected cell.

A major consideration when considering the use of systemic retroviral delivery gene therapy, both from a safety stand point and from a purely practical stand point, is as mentioned above the targeting of retroviral vectors. It is clear that therapeutic genes carried by vectors should not be indiscriminately expressed in all tissues and cells, but rather only in the requisite target cell. This is especially important if the genes to be transferred are toxin genes aimed at ablating specific tumour cells. Ablation of other, nontarget cells would obviously be very undesirable.

A number of retroviral vector systems have been previously described that should allow targeting of the carried therapeutic genes (reviewed in Salmons and Gunzburg, 1993). Most of these approaches involve either limiting the infection event to predefined cell types or using heterologous promoters to direct expression of linked heterologous therapeutic or marker genes to specific cell types. Heterologous promoters are used which should drive expression of linked genes only in the cell type in which this promoter is normally active.

In danish patent application no. 1017/94 the principle and construction of a new type of retroviral vector, the ProCon-vector, carrying various types of tissue specific regulatory elements are described:

The retroviral genome consists of an RNA molecule with the structure R-U5-gag-pol-env-U3-R. During the process of reverse transcription, the U5 region is duplicated and placed at the right hand end of the generated DNA molecule, whilst the U3 region is duplicated and placed at the left hand end of the generated DNA molecule. The resulting structure U3-R-U5 is called LTR (Long Terminal Repeat) and is thus identical and repeated at both ends of the DNA structure or provirus (Varmus, 1988). The U3 region at the left hand end of the provirus harbours the promoter (see below). This promoter drives the synthesis of an RNA transcript initiating at the boundary between the left hand U3 and R regions and terminating at the boundary between the right hand R and U5 region. This RNA is packaged into retroviral particles and transported into the target cell to be infected. In the target cell the RNA genome is again reverse transcribed as described above.

In the ProCon-vector the right-hand U3 region is altered (Fig. 1), but the normal left-hand U3 structure is maintained (Fig. 1); the vector can be normally transcribed into RNA utilizing the normal retroviral promoter located within the left-hand U3 region (Fig. 1). However the generated RNA will only contain the altered right-hand U3 structure. In the infected target cell, after reverse transcription, this altered U3 structure will be placed at both ends of the retroviral structure (Fig. 1).

If the altered region carries a polylinker (see below) instead of the U3 region then any promoter, including those directing tissue specific expression such as the WAP promoter (see below) can be easily inserted. This promoter can then be utilized exclusively in the target cell for expression of linked genes carried by the retroviral vector. Additionally DNA segments homologous to one or more celluar sequences can be inserted into the polylinker for the purposes of gene targeting, by homologous recombination.

The expression vectors used for the purpose of the invention need not be of the ProCon type, but can be any conventional vector carrying heterologous DNA sequence(s) under transcriptional control of the WAP or MMTV regulatory sequences. The vector used for the purpose of the invention is preferably a retroviral

vector of conventional type i.e. with the WAP or MMTV promoters used as internal promoters, i.e. LTR-neo-*WAP-tk*-LTR, but most preferably a retroviral vector of the ProCon type.

Vector constructs carrying various types of mammary gland specific regulatory elements has been tested in mice where expression of a marker gene driven by the regulatory elements in the hormonally stimulated mammary gland could be achieved (DK patent application no. 1017/94). One regulatory element demonstrated to give rise to expression in the pregnant and lactating mouse mammary gland is a small region of the rodent WAP promoter (Kolb et al., 1994). This gene is only expressed in the pregnant and lactating mammary glands of rodents and has no human homologue (Hennighausen, 1992). It is therefore not predictable that this regulatory element will function at all to direct expression in human cells and/or allow expression in human mammary carcinoma cells.

It was thus quite unexpected when the inventors of the present invention found that a 578bp element of the WAP promoter is able to direct expression of a linked marker gene (ß-gal) in primary human mammary carcinoma cells.

Summary of the Invention

The invention then, inter alia, comprises the following, alone or in combination:

The use of the WAP or MMTV regulatory sequences for the expression of linked heterologous DNA seguences in human mammary cells, including human mammary carcinoma cells;

the use as above wherein the regulatory sequence is the 578bp element of the WAP promoterregion or any other element/region of the WAP regulatory sequence conferring mammary specific expression;

the use as above wherein the regulatory sequence is the U3 region of MMTV or subregions thereof conferring mammary specific expression;

the use as above wherein the heterologous DNA sequence(s) is selected from the group consisting of marker genes, therapeutic genes, antiviral genes, antitumour genes, and cytokine genes;

the use as above, wherein said marker or therapeutic gene is selected from the group consisting of marker genes which codes for proteins such as ß-galactosidase, neomycin, alcohol dehydrogenase, puromycin, hypoxanthine phosphoribosyl transferase (HPRT), hygromycin and secreted alkaline phosphatase or therapeutic genes which codes for proteins such as Herpes Simplex Virus thymidine kinase, cytosine deaminase, guanine phosphoribosyl transferase (gpt), cytochrome P 450 and cell cycle regulatory genes which codes for proteins such as P.T.O., SDI or tumor supressor genes which codes for proteins such as p53 or antiproliferation genes which codes for proteins such as melittin, cecropin or cytokines such as IL-2;

the use as above, wherein the regulatory sequences and the linked heterologous DNA sequences form a part of a retroviral provirus integrated in the genome of the human mammary cell, including human mammary carcinoma cell;

the use as above, wherein the provirus in the infected cell has a 5' LTR region comprising a completely or partially deleted U3 region wherein said deleted region has been replaced by a polylinker containing the WAP or MMTV regulatory sequences followed by the R and U5 region; one or more sequences selected from non coding sequences and coding sequences; and a 3' LTR comprising completely or partially deleted U3 region wherein said deleted region has been replaced by a polylinker containing the WAP or MMTV regulatory sequences followed by the R and U5 region;

a retroviral provirus integrated in the human genome carrying a DNA-construct comprising one or more heterologous DNA-sequences under transcriptional control of the WAP or MMTV regulatory sequences;

a retroviral provirus as above characterised by a 5' LTR region comprising a completely or partially deleted U3 region wherein said deleted region has been replaced by a polylinker containing the WAP or MMTV regulatory sequences followed by the R and U5 region; one or more sequences selected from non coding sequences and coding sequences; and a 3' LTR comprising completely or partially deleted U3 region wherein said deleted region has been replaced by a polylinker containing the WAP or MMTV regulatory sequences followed by the R and U5 region;

a human mammary cell, including a human mammary carcinoma cell, containing a DNA construct carrying one or more heterologous DNA-sequences under transcriptional control of the WAP or MMTV regulatory sequences;

a cell as above, wherein the regulatory sequence is the 578bp element of the WAP promoterregion or any other element/region of the WAP regulatory sequence conferring mammary specific expression;

a cell as above, wherein the regulatory sequence is the U3 region of MMTV or subregions thereof conferring mammary specific expression,

a cell as above, wherein the heterologous DNA sequence(s) is selected from the group consisting of marker genes, therapeutic genes, antiviral genes, antitumour genes, and cytokine genes;

a cell as above, wherein said marker or therapeutic gene is selected from the group consisting of marker genes which codes for proteins such as ß-galactosidase, neomycin, alcohol dehydrogenase, puromycin, hypoxanthine phosphoribosyl transferase (HPRT), hygromycin and secreted alkaline phosphatase or therapeutic genes which codes for proteins such as Herpes Simplex Virus thymidine kinase, cytosine deaminase, guanine phosphoribosyl transferase (gpt), cytochrome P 450 and cell cycle regulatory genes which codes for proteins such as P.T.O., SDI or tumor supressor genes which codes for proteins such as p53 or antiproliferation genes which codes for proteins such as melittin, cecropin or cytokines such as IL-2;

a cell as above, wherein the DNA construct form a part of a retroviral provirus integrated in the genome of the cell;

a cell as above, wherein the provirus have a 5' LTR region comprising a completely or partially deleted U3 region wherein said deleted region has been replaced by a polylinker containing the WAP or MMTV regulatory sequences followed by the R and U5 region; one or more sequences selected from non coding sequences and coding sequences; and a 3' LTR comprising completely or partially deleted U3 region wherein said deleted region has been replaced by a polylinker containing the WAP or MMTV regulatory sequences followed by the R and U5 region;

a human packaging cell line or a packaging cell line histocompatible with human tissue harbouring

1) a retroviral vector carrying a DNA construct comprising one or more heterologous DNA-sequences under transcriptional control of the WAP or MMTV regulatory sequences, and

2) at least one retroviral or recombinant retroviral construct coding for proteins required for said retroviral vector to be packaged;

a cell line as above wherein the retroviral vector comprises a 5' LTR region of the structure U3-R-U5; one or more sequences selected from coding and non-coding sequences; and
a 3' LTR region comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a polylinker sequence containing the WAP or MMTV regulatory sequences, followed by the R and U5 region;

a cell line as above, wherein the regulatory sequence is the 578bp element of the WAP promoterregion or any other element/region of the WAP regulatory sequence conferring mammary specific expression;

a cell line as above, wherein the regulatory sequence is the U3 region of MMTV or subregions thereof conferring mammary specific expression;

a cell line as above, wherein the heterologous DNA sequence(s) is selected from the group consisting of marker genes, therapeutic genes, antiviral genes, antitumour genes, and cytokine genes;

a cell line as above, wherein said marker or therapeutic gene is selected from the group consisting of marker genes which codes for proteins such as ß-galactosidase, neomycin, alcohol dehydrogenase, puromycin, hypoxanthine phosphoribosyl transferase (HPRT), hygromycin and secreted alkaline phosphatase or therapeutic genes which codes for proteins such as Herpes Simplex Virus thymidine kinase, cytosine deaminase, guanine phosphoribosyl transferase (gpt), cytochrome P 450 and cell cycle regulatory genes which codes for proteins such as P.T.O., SDI or tumor

supressor genes which codes for proteins such as p53 or antiproliferation genes which codes for proteins such as melittin, cecropin or cytokines such as IL-2;

a method for the treatment of breast cancer comprising administering to a human an expression targeted vector carrying one or more genes selected from the group consisting of therapeutic genes, antitumour genes, and cytokine genes under transcriptional control of the WAP or MMTV regulatory sequences; either as recombinant vector virus particle or recombinant vector virus producing cells.

The WAPgal and the MMTVgal constructs are of particular interest for the purposes of the present invention because the regulatory elements conferring tissue specificity are both derived from the rodent system. This may become an important safety feature because the use of human regulatory sequences in a retroviral vector could cause problems because homologous recombinations between the vector carried sequences and the corresponding cellular may cause genome instability (PTO).

The retroviral vector is based preferably either on a BAG vector (Price *et al.*, 1987) or an LXSN vector (Miller and Rosman, 1989).

The coding sequence is preferably selected from one or more elements of the group consisting of marker genes, therapeutic genes, antiviral genes, antitumour genes, cytokine genes.

Said marker and therapeutic genes are preferably selected from the group consisting of marker genes which codes for proteins such as ß-galactosidase, neomycin, alcohol dehydrogenase, puromycin, hypoxanthine phosphoribosyl transferase (HPRT), hygromycin and secreted alkaline phosphatase or therapeutic genes which codes for proteins such as Herpes Simplex Virus thymidine kinase, cytosine deaminase, guanine phosphoribosyl transferase (gpt), cytochrome P 450 and cell cycle regulatory genes which codes for proteins such as P.T.O., SDI or tumor supressor genes which codes for proteins such as p53 or antiproliferation genes which codes for proteins such as melittin, cecropin or cytokines such as IL-2.

The packaging cell line is preferably selected from an element of the group consisting of psi-2, psi-Crypt, psi-AM, GP+E-86, PA317 and GP+envAM-12, or of any of these transfected with recombinant constructs allowing expression of surface proteins from other enveloped viruses.

A further embodiment of the invention provides non-therapeutical method for introducing homologous and/or heterologous nucleotide sequences into human cells *in vitro* and *in vivo* comprising transfecting a packaging cell line of a retroviral vector system with a retroviral vector carrrying one or more heterologous DNA sequences under transcriptional control of the Wap or MMTV regulatory sequences and infecting a target cell population with recombinant retroviruses produced by the packaging cell line. The heterologous DNA sequences are selected from one or more elements of the group consisting of genes or parts of genes encoding for proteins, regulatory sequences and promoters.

According to the invention the term "polylinker" is used for a short stretch of artificially synthesized DNA which carries a number of unique restriction sites allowing the easy insertion of any promoter or DNA segment. The term "heterologous" is used for any combination of DNA sequences that is not normally found intimately associated in nature.

The following example will illustrate the invention further. The example is however in no way intended to limit the scope of the present invention as obvious modifications will be apparent, and still other modifications and substitutions will be apparent to anyone skilled in the art.

The recombinant DNA methods employed in practicing the present invention are standard procedures, well known to those skilled in the art, and described in detail, for example, in "Molecular Cloning" (Sambrook et al. 1989) and in "A Practical Guide to Molecular Cloning" (Perbal, 1984).

Example 1

Mammary gland specific expression after infection with ProCon Vectors carrying mammary specific promoters.

In the murine leukemia virus (MLV) retroviral vector known as BAG (Price et al., 1987) the ß-galactosidase gene is driven by the promiscuous (i.e. non-tissue specific) MLV promoter in the U3 region of the LTR (Fig. 1). According to the present invention

a derivative of the BAG vector has been constructed in which the MLV promoter (U3) located within the 3'LTR (Fig. 1) has been deleted by PCR. At this position a polylinker was inserted containing the restriction sites *Sac*I and *Mlu*I allowing the facile introduction of heterologous promoters. The BAG vector lacking the U3 is expressed from the MLV promoter (U3) within the 5'LTR when introduced into a packaging cell line. As a result of the rearrangements occurring in the retroviral genome during its life cycle, following infection of its target cell, the polylinker will be duplicated at both ends of the retroviral genome as described in danish patent application no. 1017/94. Thereby a retroviral vector can be constructed in which the expression of the ß-galactosidase gene of BAG will be controlled by any heterologous promoter inserted into the polylinker in the target cell (Fig. 1).

According to the principle set forth above the following specific promoters have been inserted into the polylinker region or the modified BAG vector:

The Mouse Mammary Tumour Virus (MMTV) U3-Region (mtv-2) without the inverted repeats, and several subregions of the MMTV promoter including a region that confers responsiveness to glucocorticoid hormones and a region containing an element that directs expression to the mammary gland.

The Whey Acidic Protein (WAP) promoterregion encompassing the positions -447 to +131 (with the transcription initiation site defined as +1), the 578 bp element, controls the expression of WAP so that it is only produced in the mammary glands of pregnant and lactating rodents.

The control of the ß-galactosidase gene expression by promoters inserted into the polylinker has been validated by infection studies using the constructed MMTV and WAP retroviral vectors to infect various cells.

To produce retroviral vector particles, the MMTV and WAP ProCon vectors have been transfected into the packaging cell line GP+E86 (Markowitz et al., 1988). After selection for neomycin resistance, which is encoded by the vector, stable populations and clones of recombinant ProCon virus producing cells were obtained. Virus containing supernatant from these populations was used to infect explanted normal primary human mammary tissue obtained from reduction mammaplasties. Since it is known that these promoters are responsive to pregnancy hormones, the tissue was

cultivated in the presence of such hormones. The expression of the marker gene was determined by a quantitative ß-gal assay which is based on the detection of ß-galactosidase activity by chemiluminescence. In all the experiments the original, non-tissue specific BAG-vector was used as a positive control. All of the analysed samples showed ß-galactosidase expression (Fig. 2) in three independent experiments. It has thus been demonstrated for the first time that the WAP regulatory elements as well as the MMTV-U3 region can drive the expression of a gene within a MLV retroviral vector in primary human mammary gland cells.

To determine whether these regulatory sequences are active in human mammary tumours as well, primary explants of human mammary tumours were infected with WAPgal. A few days later the tumour organoids were analysed for ß-gal expression as in the experiments described above. In this experiment the human mammary tumour cells infected with the WAPgal retroviral vector showed ß-gal expression (Fig. 3a). In another experiment it was demonstrated that the MMTVgal (125.gal) and the non-tissue specific BAG construct also express the ß-gal gene in primary normal human mammary cells(i.e. non tumor derived cells) (Fig. 3b).

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CLAIMS

1. Use of the WAP or MMTV regulatory sequences for the expression of linked heterologous DNA seguences in human mammary cells, including human mammary carcinoma cells.

2. Use according to claim 1 wherein the regulatory sequence is the 578bp element of the WAP promoterregion or any other element/region of the WAP regulatory sequence conferring mammary specific expression.

3. Use according to claim 1 wherein the regulatory sequence is the U3 region of MMTV or subregions thereof conferring mammary specific expression.

4. Use as in claim 1 wherein the heterologous DNA sequence(s) is selected from the group consisting of marker genes, therapeutic genes, antiviral genes, antitumour genes, and cytokine genes.

5. Use as in claim 4, wherein said marker or therapeutic gene is selected from the group consisting of marker genes which codes for proteins such as ß-galactosidase, neomycin, alcohol dehydrogenase, puromycin, hypoxanthine phosphoribosyl transferase (HPRT), hygromycin and secreted alkaline phosphatase or therapeutic genes which codes for proteins such as Herpes Simplex Virus thymidine kinase, cytosine deaminase, guanine phosphoribosyl transferase (gpt), cytochrome P 450 and cell cycle regulatory genes which codes for proteins such as P.T.O., SDI or tumor supressor genes which codes for proteins such as p53 or antiproliferation genes which codes for proteins such as melittin, cecropin or cytokines such as IL-2.

6. Use as in claim 1, wherein the regulatory sequences and the linked heterologous DNA sequences form a part of a retroviral provirus integrated in the genome of the human mammary cell, including human mammary carcinoma cell.

7. Use as in claim 6 wherein the provirus have a 5' LTR region comprising a completely or partially deleted U3 region wherein said deleted region has been replaced by a polylinker containing the WAP or MMTV regulatory sequences followed by the R and U5 region; one or more sequences selected from non coding sequences and coding sequences; and a 3' LTR comprising completely or partially

deleted U3 region wherein said deleted region has been replaced by a polylinker containing the WAP or MMTV regulatory sequences followed by the R and U5 region.

8. A retroviral provirus integrated in the human genome carrying a DNA-construct comprising one or more heterologous DNA-sequences under transcriptional control of the WAP or MMTV regulatory sequences.

9. Retroviral provirus integrated in the human genome characterised by a 5' LTR region comprising a completely or partially deleted U3 region wherein said deleted region has been replaced by a polylinker containing the WAP or MMTV regulatory sequences followed by the R and U5 region; one or more sequences selected from non coding sequences and coding sequences; and a 3' LTR comprising completely or partially deleted U3 region wherein said deleted region has been replaced by a polylinker containing the WAP or MMTV regulatory sequences followed by the R and U5 region.

10. A human mammary cell, including a human mammary carcinoma cell, containing a DNA construct carrying one or more heterologous DNA-sequences under transcriptional control of the WAP or MMTV regulatory sequences.

11. A human packaging cell line or a packaging cell line histocompatible with human tissue harbouring

1) a retroviral vector carrying a DNA construct comprising one or more heterologous DNA-sequences under transcriptional control of the WAP or MMTV regulatory sequences, and

2) at least one retroviral or recombinant retroviral construct coding for proteins required for said retroviral vector to be packaged.

13. A method for the treatment of breast cancer comprising administering to a human an expression targeted vector carrying one or more genes selected from the group consisting of therapeutic genes, antitumour genes, and cytokine genes under transcriptional control of the WAP or MMTV regulatory sequences; either as recombinant vector virus particle or recombinant vector virus producing cells.

Abstract

The present invention relates to the use of the WAP or MMTV regulatory sequences for the targeted expression of linked heterologous DNA seguences in human mammary cells, including human mammary carcinoma cells.

Construction of a U3 minus BAG-vector (MLV)

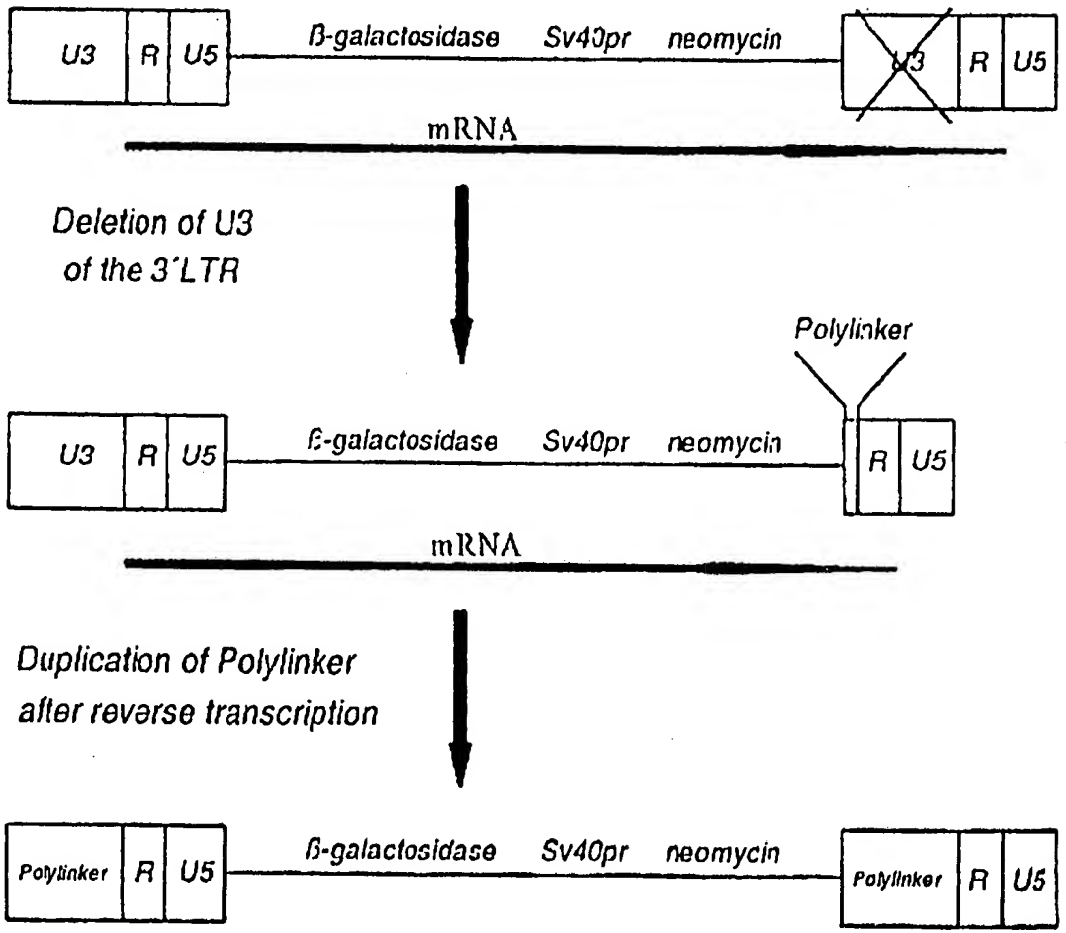

FIG 1

ß-Gal Expression of vector contructs after infection of primary hum. mgl cells

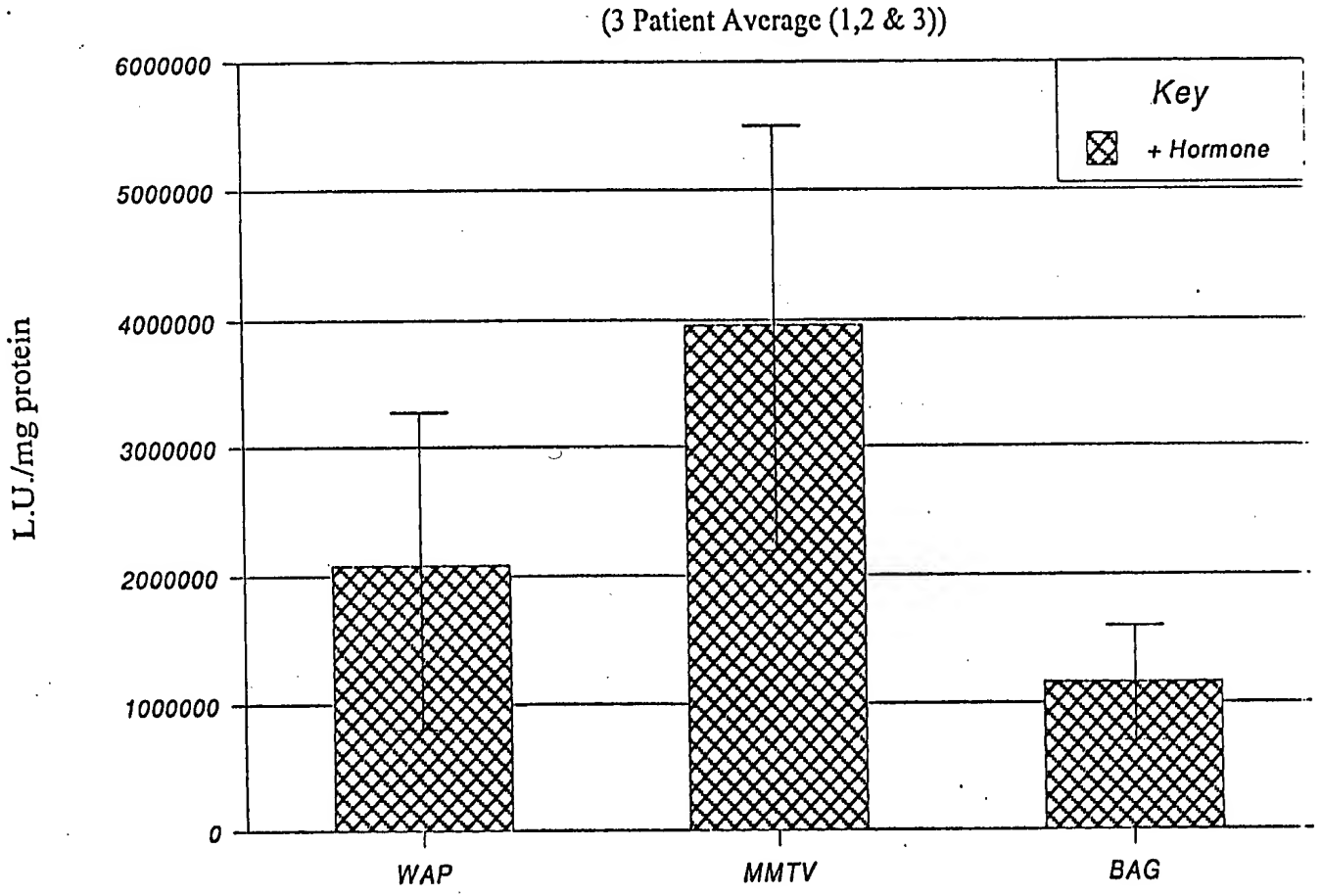

FIG 2